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FEMS Microbiology Letters 148 (1997) 53–57

FEMS MICROBIOLOGY LETTERS

Actinobacillus pleuropneumoniae does not require urease activity to produce acute swine pleuropneumonia

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Received 19 December 1996; accepted 10 January 1997

Abstract

The role in virulence of *Actinobacillus pleuropneumoniae* urease activity was investigated. A urease-negative mutant was isolated following transposon mutagenesis with a mini-Tn*10* derivative. Both the parent strain and the urease-negative mutant exhibited identical LD_{50} values in a murine infection model. Pig challenge confirmed that the urease-negative mutant was fully virulent, since experimental inoculation with 5×10^7 colony forming units resulted in an acute disease indistinguishable from that produced by the wild-type strain at the same dose. Our results demonstrate that urease activity is not required for the development of acute pleuropneumonia.

Keywords: *Actinobacillus pleuropneumoniae*; Urease; Transposon mutagenesis; Virulence; Swine pleuropneumonia

1. Introduction

Actinobacillus pleuropneumoniae is a Gram-negative, facultatively anaerobic, encapsulated bacterium of the *Pasteurellaceae* family. It is the aetiological agent of porcine pleuropneumonia, a respiratory disease found worldwide and which has major economic implications for the swine industry. The disease occurs as acute outbreaks with high mortality in 24–48 h or as a chronic persistent disease. Typical lesions include necrotic and haemorrhagic pneumonia with fibrinous pleural adhesions. *A. pleuropneumoniae* is highly contagious and infects animals in intensive farming conditions. Currently available vaccines provide only poor protection, thus the development of efficient tools for control and prevention of the disease remains as a major objective in veterinary research [1]. Considerable effort has been devoted to understand the pathogenesis of the infection. Nevertheless, the mechanisms involved in the host-bacterium interaction are only partially known, and the factors responsible for many phenomena during porcine pleuropneumonia remain unclear [2].

A. pleuropneumoniae produces a potent urease activity which may be involved in virulence. Microbial ureases are key virulence factors for several urinary [3,4] and gastric pathogens [5]. Interestingly, urease

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PII S0378-1097(97)00012-8

activity has also been suggested to be involved in virulence of the respiratory pathogens *Bordetella bronchiseptica* [6] and *Mycobacterium tuberculosis* [7], as well as *Ureaplasma urealyticum*, also implicated in respiratory disease [8]. We therefore investigated whether urease activity has a role in *A. pleuropneumoniae* infection. We describe here the virulence of a transposon-induced urease-negative mutant compared to that of the wild-type strain, in the natural host and a mouse model of infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. pleuropneumoniae CM5-Nx^R, a nalidixic acid-resistant spontaneous mutant derived from strain CM5 (serotype 1) [9], was used as parent strain for transposon mutagenesis. *Escherichia coli* S17-1 (λ*pir*) [9] was used as the donor of mini-Tn*10*. Bacteria were routinely cultured at 37°C on tryptic soy broth or agar supplemented for *A. pleuropneumoniae* with 6 mg ml^{-1} of yeast extract and 10 μg ml^{-1} of NAD. Inocula for virulence assays were prepared by 6 h of culture on PPLO agar supplemented with 50 μl ml^{-1} of sterile horse serum and 10 μg ml^{-1} of NAD. Antibiotics were added to the culture media as appropriate, at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}, and nalidixic acid, 60 μg ml^{-1}.

2.2. Transposon mutagenesis and screening for urease-negative mutants

Mini-Tn*10 A. pleuropneumoniae* transposon mutagenesis was performed with the suicide vector pLOF/Km as previously described [9]. Counterselection plates containing nalidixic acid and kanamycin were cultured for 36 h at 37°C and the bacterial colonies obtained were screened for urease activity with a semi-solid (0.4% agar) overlay of urease determination medium (Difco). Individual colonies unable to alkalinise the medium after 1–3 h at 37°C were recovered and assayed again in closed microtubes using a heavily inoculated urea rapid broth (Difco). Isolates which failed to change the colour of the medium after prolonged incubation (36 h) were considered to be urease-negative mutants. The number of copies of the transposon present in each mutant was determined as previously described [9].

2.3. Virulence studies in mice

Each group of five female NMRI mice (5–6 weeks old, 20–25 g) was inoculated intraperitoneally with 0.5 ml of one of a series of fivefold dilutions of bacterial suspensions in sterile phosphate buffered saline (PBS). LD_{50} values were estimated according to the method of Reed and Muench [10]. Results were calculated as mean values (± standard deviations) from three independent experiments. Statistical significance of the LD_{50} results was determined by analysis of variance of mean values.

2.4. Virulence studies in pigs

Six-week-old (20 ± 2 kg) Large White male pigs were obtained from the experimental station farm of the University of León, where no previous clinical history of porcine pleuropneumonia has been reported. The pigs were seronegative against all the serotypes of *A. pleuropneumoniae* in tests prior to challenge. Animals were housed in isolated rooms and were fed non-medicated feed and water ad libitum. The inoculum was accurately adjusted as described [11], and 5×10^7 CFU of the mutant or parent strain were given intratracheally to each animal. Control pigs received the same volume of sterile PBS. During manipulations, the animals were tranquilised with azaperone (Stresnil®). After the experiments, swabs were taken from lungs and cultured to confirm the presence and phenotype of the inoculated strains.

All experiments conformed to the International Guiding Principles for Biomedical Research Involving Animals. The research project was approved by the Council of the Animal Health Department, University of León.

3. Results and discussion

Five transposon insertion mutants with urease-negative phenotype were obtained by a two-step screening protocol. First, transconjugants obtained

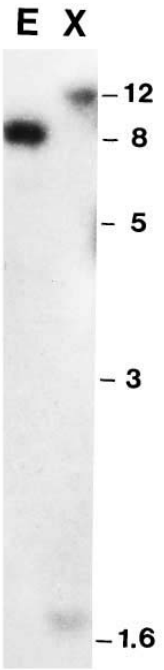

Fig. 1. Southern blot analysis of chromosomal DNA from the urease-negative mutant CM5-U$^-$ digested with *Eco*RI (E), and *Xho*I (X). Chromosomal DNA from the parent strain CM5-Nx^R did not hybridise with the probe (not shown). Numbers indicate the positions of markers and their sizes in kb.

from mini-Tn*10* insertional mutagenesis were tested for urease activity by overlay screening. This procedure identified most of the urease-positive colonies, but did not allow unequivocal detection of urease-negative mutants, because the modification in the colour of the medium due to the numerous urease-positive colonies masked the urease-negative phenotype within hours. Colonies showing an altered alkalinisation of the medium were then retested independently in closed microtubes such that urease-negative mutants were unambiguously identified. The five mutants were kanamycin-resistant and ampicillin-sensitive, indicating that they resulted from transposition and not from plasmid co-integration. Chromosomal DNA from each mutant was digested with *Eco*RI (which does not cut the transposon) and *Xho*I (which cuts the transposon once) and probed for hybridisation by Southern blotting with radiolabelled pLOF/Km (the delivery vector for the mini-Tn*10*) to assess the number of copies of the transposon present. Four mutants carried single transposon insertions, and one of them, designated CM5-U$^-$ (Fig. 1), was selected for subsequent in vivo virulence experiments.

An experimental infection model in mice, based on LD_{50} determinations after intraperitoneal inoculation, has previously been successfully used to detect serotype-related variations in *A. pleuropneumoniae* virulence [12], and to demonstrate loss of virulence ($\sim$20-fold increase the LD_{50}) in a haemolysin-deficient mutant [13]. Therefore, we used the same approach to determine the effect of urease activity on *A. pleuropneumoniae* virulence.

No significant difference ($P < 0.001$) between the LD_{50} of the parent strain CM5-Nx^R, $3.20(\pm 1.95)\times 10^7$, and that of the urease-negative mutant CM5-U$^-$, $2.97(\pm 2.11)\times 10^7$, was observed in this mouse model. Thus, loss of the ureolytic character in *A. pleuropneumoniae* is not associated with poorer virulence in mice.

A. pleuropneumoniae has a strict host specificity for the pig. Failure to demonstrate any effect on virulence by experimental infection in mice could have been due to the insensitivity of the animal model. Therefore, virulence experiments were also performed in the natural host for *A. pleuropneumoniae*.

Pigs were experimentally inoculated with the *A. pleuropneumoniae* urease mutant or its parent strain. The animals infected with the CM5-U$^-$ mutant presented a peracute swine pleuropneumonia syndrome, indistinguishable from that produced by the parent strain CM5-Nx^R. One animal inoculated with the strain CM5-U$^-$ died 6 h after it was challenged, and the other five died within 12–16 h post-inoculation, showing similar symptoms and haemorrhagic lung lesions.

We did not characterise the *locus* disrupted by the transposon in our urease-negative mutant. Therefore, in addition to the genetic determinant responsible for the urease-negative phenotype, we cannot exclude that other genes have been affected by the mini-Tn*10* insertion due to polar or pleiotropic effects. This would have posed problems of interpretation if we had found a difference in virulence following loss of the ureolytic character. Nevertheless, since we did not observe any effect, the involvement of urease activity in the virulence of *A. pleuropneumoniae* can be ruled out, at least under the experimental conditions studied.

Our findings are consistent with those for some other microbial ureases. For example, the presence or absence of urease in *B. bronchiseptica* does not affect pathogenesis [6]. Similarly, urease activity is not essential for the persistence of *Mycobacterium bovis* BCG in cultured macrophages or in mice [14].

Nevertheless, there were reasons to believe that *A. pleuropneumoniae* urease activity may have been involved in the host-bacterium interaction. *A. pleuropneumoniae* cannot live saprophytically, and its only environmental niche is the respiratory tract of the pig. Most of the non-pathogenic members of the V factor-dependent resident microflora of the porcine upper respiratory tract, namely *Actinobacillus porcinus*, *Actinobacillus indolicus*, and *Haemophilus* taxon C, are urease-negative [15]. Indeed, ureolytic activity has an important role in pathogenesis in many bacterial infections, which might have been expected to be parallelled in some way by *A. pleuropneumoniae*. *H. pylori* and *P. mirabilis* ureases have cytotoxic effects in vitro on gastric adenocarcinoma cells [16] and renal epithelial cells [17], respectively. *A. pleuropneumoniae* adheres to epithelial cells of the lower respiratory tract, and this may be the first step in pathogenesis [18]. Possibly ammonium hydroxide generated by ureolytic activity is a toxic factor in this intimate association. It was recently reported that Apx toxins are able to kill type II alveolar epithelial cells [19], a finding which does not exclude a contribution of urease activity in the toxicity to eukaryotic cells. This could not be easily verified however, due to the expected masking effect of Apx toxins. Moreover, urease activity has been argued to contribute to virulence by impeding the deleterious effect of low pH in phagosomes both in *M. tuberculosis* [7] and in *Yersinia enterocolitica* [20]. Therefore the ability of *A. pleuropneumoniae* to survive within porcine neutrophils and alveolar macrophages [11,21] may be partially explained by the urease activity. Again, the major contribution of Apx toxins to the antiphagocytic activity of *A. pleuropneumoniae* [22,23] makes it difficult to determine whether or not urease activity is also involved in overcoming the hostile environment of the endocytic vacuole.

In summary, we have shown that urease activity is not a virulence factor needed by *A. pleuropneumoniae* to cause acute disease, a finding in agreement with the report of a urease-negative variant of *A. pleuropneumoniae* that was responsible for a swine pleuropneumonia outbreak [24]. However, the murine model of infection with *A. pleuropneumoniae* is only useful to study the acute phase of porcine pleuropneumonia [12]. Similarly, the experimental inoculation of pigs with a large dose of bacteria causes acute disease in which highly active virulence determinants may mask the contribution of other factors. Thus, we cannot exclude the involvement of *A. pleuropneumoniae* urease in the pathogenesis of chronic infection, or a more subtle role of the enzyme in virulence. Experiments are currently in progress in our laboratory to determine the molecular basis of the urease-negative phenotype, as well as the possible contribution of the *A. pleuropneumoniae* urease to chronic infection.

Acknowledgments

We thank Laboratorios Ovejero S.A. (León, Spain) and E. Martínez for their assistance in pig challenge experiments, and to Antibióticos S.A. for providing us with mice. We are indebted to M. Simonet for critical reading of the manuscript, and to P. Berche for friendly support and constant encouragement. This work received financial support from the 'Programa Nacional de Investigación y Desarrollo Ganadero' Grant GAN89-0317 (CICYT) to E.F.R.-F., and from Grant PB94-0330 (DGICYT) to J.-A.V.-B.

References

[1] Nicolet, J. (1992) *Actinobacillus pleuropneumoniae*. In: Diseases of Swine (Leman, A.D., Straw, B., Mengeling, W., D'Allaire, S. and Taylor D.J., Eds.), 7th edn., pp. 401–408. Iowa State University Press, Ames, IA.

[2] Tascón, R.I., Vázquez-Boland, J.A., Gutiérrez-Martín, C.B., Rodríguez-Barbosa, I. and Rodríguez-Ferri, E.F. (1996) Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*. Microbiología SEM 12, 171–184.

[3] Gatermann, S. and Marre, R. (1989) Cloning and expression of *Staphylococcus saprophyticus* urease gene sequences in *Staphylococcus carnosus* and contribution of the enzyme to virulence. Infect. Immun. 57, 2998–3002.

[4] Mobley, H.L.T. and Belas, R. (1995) Swarming and pathoge-

nicity of *Proteus mirabilis* in the urinary tract. Trends Microbiol. 3, 280–284.
[5] Lee, A., Fox, J. and Hazell, S. (1993) Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. 61, 1601–1610.
[6] Monack, D.M. and Falkow, S. (1993) Cloning of *Bordetella pertussis* urease genes and analysis of colonization by a urease-negative mutant strain in a guinea-pig model. Mol. Microbiol. 10, 545–553.
[7] Clemens, D.L., Lee, B.-Y. and Horwitz, M.A. (1995) Purification, characterization, and genetic analysis of *Mycobacterium tuberculosis* urease, a potentially critical determinant of host-pathogen interaction. J. Bacteriol. 177, 5644–5652.
[8] Rudd, P.T., Cassell, G.H., Waites, K.B., Davis, J.K. and Duffy, L.B. (1989) *Ureaplasma urealyticum* pneumonia: experimental production and demonstration of age-related susceptibility. Infect. Immun. 57, 918–925.
[9] Tascón, R.I., Rodríguez-Ferri, E.F., Gutiérrez-Martín, C.B., Rodríguez-Barbosa, I., Berche, P. and Vázquez-Boland, J.A. (1993) Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn*10* derivative. J. Bacteriol. 175, 5717–5722.
[10] Reed, L.J. and Muench, H. (1938) A simple method for estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.
[11] Cruijsen, T.L.M., van Leengoed, L.A.M.G., Dekker-Nooren, T.C.E.M., Schoevers, E.J. and Verheijden, J.H.M. (1992) Phagocytosis and killing of *Actinobacillus pleuropneumoniae* by alveolar macrophages and polymorphonuclear leukocytes isolated from pigs. Infect. Immun. 60, 4867–4871.
[12] Tascón, R.I., Vázquez-Boland, J.A., Gutiérrez-Martín, C.B., Rodríguez-Barbosa, I. and Rodríguez-Ferri, E.F. (1994) Utilidad del modelo murino para el estudio de la pleuroneumonía porcina. An. Fac. Vet. León 38, 107–118
[13] Tascón, R.I., Vázquez-Boland, J.A., Gutiérrez-Martín, C.B., Rodríguez-Barbosa, I. and Rodríguez-Ferri, E.F. (1994) The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. Mol. Microbiol. 14, 207–216.
[14] Reyrat, J.-M., Lopez-Ramirez, G., Ofredo, C., Gicquel, B. and Winter, N. (1996) Urease activity does not contribute dramatically to persistence of *Mycobacterium bovis* bacillus Calmette-Guérin. Infect. Immun. 64, 3934–3936.
[15] Møller, K., Fussing, V., Grimont, P.A.D., Paster, B.J., Dewhirst, F.E. and Kilian, K. (1996) *Actinobacillus minor* sp. nov., *Actinobacillus porcinus*, sp. nov., and *Actinobacillus indolicus* sp. nov., three new V factor-dependent species from the respiratory tract of pigs. Int. J. Syst. Bacteriol.46, 951–956.
[16] Smoot, D.T., Mobley, H.L.T., Chippendale, G.R., Lewison, J.F. and Resau, J.H. (1990) *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. 58, 1992–1994.
[17] Mobley, H.L.T., Chippendale, G.R., Swihart, K.G. and Welch, R.A. (1991). Cytotoxicity of the HpmA hemolysin and urease of *Proteus mirabilis* and *Proteus vulgaris* against cultured human renal proximal tubular epithelial cells. Infect. Immun. 59, 2036–2042.
[18] Dom, P., Haesebrouck, F., Ducatelle, R. and Charlier, G. (1992) In vivo association of *Actinobacillus pleuropneumoniae* serotype 2 with the respiratory epithelium of pigs. Infect. Immun. 62, 1262–1267.
[19] van de Kerkhof, A., Haesebrouck, F., Chiers, K., Ducatelle, R., Kamp, E.M. and Smits, M.A. (1996) Influence of *Actinobacillus pleuropneumoniae* and its metabolites on porcine alveolar epithelial cells. Infect. Immun. 64, 3905–3907.
[20] de Koning-Ward, T.F. and Robins-Browne, R.M. (1995) Contribution of urease to acid tolerance in *Yersinia enterocolitica*. Infect. Immun. 63, 3790–3795.
[21] Udeze, F.A. and Kadis, S. (1992) Effects of *Actinobacillus pleuropneumoniae* hemolysin on porcine neutrophil function. Infect. Immun. 60, 1558–1567.
[22] Jansen, R., Briaire, J., Smith, H.E., Dom, P., Haesebrouck, F., Kamp, E.M., Gielkens, A.L.J. and Smits, M.A. (1995) Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. Infect. Immun. 63, 27–37.
[23] Tarigan, S., Slocombe, R.F., Browning, G.F. and Kimpton, W. (1994) Functional and structural changes of porcine alveolar macrophages induced by sublytic doses of a heat-labile, hemolytic, cytotoxic substance produced by *Actinobacillus pleuropneumoniae*. Am. J. Vet. Res. 55, 1548–1557.
[24] Blanchard, P.C., Walker, R.L. and Gardner, I. (1993) Pleuropneumonia in swine associated with a urease-negative variant of *Actinobacillus pleuropneumoniae* serotype 1. J. Vet. Diagn. Invest. 5, 279–282.